REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	28 Feb 90	Final 1 July 89–1 Jan 90

4. TITLE AND SUBTITLE

A BIOLOGICAL MODEL OF THE EFFECTS OF TOXIC SUBSTANCES

5. FUNDING NUMBERS

F49620-89-C-0099

6. AUTHOR(S)

AD-A221 131

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AFOSR·TR· 90·0517

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

400.4TC89

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

ASAF, AFSC
Air Force Office of Scientific Research
Bldg. 410
Bolling AFB D.C. 20332-6448

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

61102F

3005/A1

NL

DTIC ELECTE S D

11. SUPPLEMENTARY NOTES

Best Available Copy

12a. DISTRIBUTION/AVAILABILITY STATEMENT

unlimited

12b. DISTRIBUTION CODE

D 3

13. ABSTRACT (Maximum 200 words)

Due to the basic nature of military operations, it is sometimes necessary for Air Force personnel to be exposed to toxic chemicals in their work environment either as a protracted low-level exposure or as a high-level, acute exposure.

The application herein proposes to use the anterior eye (cornea and conjunctiva) and its sensory innervation as an assay of toxic effects. The anterior eye has unique characteristics: its extensive use in toxicology, permeability, the relative ease of observation, and importance to the input of visual information. In addition, the eye is unique in that receptors innervating the cornea differ from those innervating conjunctiva and hence comparisons of these populations will likely be useful to assay for deficits in performance of different types of sensory neurons.

The personnel of Topical Testing have expertise in growing (and recording from) neural tissue in culture, as well as the design and fabrication of specialized equipment used in such studies. This phase I feasibility study will investigate the use of an ocular-neuronal model as an indicator of toxic insult. Phase II development will establish a model tissue culture system with an eventual goal of investigating mechanisms to enhance natural detoxification. (KR)

14. SUBJECT TERMS

Bioassay, Environmental Toxins, Detoxification, Tissue Culture

15. NUMBER OF PAGES

45

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

Standard Form 298 (Rev. 2-89)

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LIST OF ABBREVIATIONS

For the convenience of the reader, below is a list of abbreviations used in the text of this proposal.

SPS   —   Standard Pollutant Stimulus

Introduction

As discussed above, the traditional test for toxicity and irritation is the Draize test in which the chemicals are placed in the eye in an awake rabbit and the effects of these substances are monitored over a 21 days period. In recent years the Draize test has been increasingly criticized; and as a consequence, more tissue culture assays have been proposed to complement, and perhaps replace, different aspects of the Draize assay.

Topical Testing has proposed a model for the neural component of the ocular response. Phase I has focused on (1) confirming that published protocols are adequate to obtain cultures of corneal and conjunctival epithelium, (2) obtaining co-cultures with neurons, and (3) improving established protocols. In addition, it was necessary to investigate various potential problems with the tissue culture/neural recording system with the eventual goal of (4) developing an integrated commercial package (see below, Commercial Market Assessment). Furthermore, it was found that (5) specialized data acquisition hardware and software was highly desirable in such a system.

Another goal was (6) to record from single sensory neurons in culture to determine whether their responses to hyperpolarizing and depolarizing currents were similar to those obtained in other preparations. Lastly, (7) Topical Testing has chosen an environmental pollutant to which the neurons in culture were exposed via a (8) micro-pressure injection system. Pressure injection has advantages over a general perfusion system in that the injection concentrates the solution in the micro area surrounding the nerve being recorded from and hence limits to a great extent the exposure of the rest of the tissue culture to the pollutant. Therefore, it is possible to record from a number of neurons within the culture dish without exposing them to a significant level of toxic material. In this way, toxicity of substances can be efficiently screened on a number of neurons in a single culture. Because of the wealth of data that has been accumulated using the Draize test over the past forty years, it would be useful for the proposed rat model to be compared to the analogous rabbit model. Such experiments are proposed in the phase II application outlined below.

One advantage of tissue culture over an in vivo preparation is that chronic exposures can be obtained in tissue culture with a controlled dose of pollutant over a specified time period. For example, the neurons can be grown for a specified number of days and then chronically exposed to the toxin at a low level before neural recording takes place. Other designs would include an acute exposure (analogous to the Draize test) followed by recordings over a period of time following the acute exposure. Preliminary experiments have been that investigate the possibility of repeated recording from the same neurons in culture. Furthermore, analogous in vivo preparation would be one in which the animal is exposed to low levels of toxins then the animal is sacrificed and its neurons placed in tissue culture, and monitored to determine whether there are residual effects of toxic exposure that are retained by the neurons in culture. To this end we have conducted experiments demonstrating that neurons from adult animals can be cultured.

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An additional advantage of tissue culture is that neurons can be visualized and studied in isolation, allowing analysis of the cellular process of toxicological insult in greater detail than otherwise possible. Once the details of the influence of individual pollutants on neural function are known, it will be possible to investigate means of reducing or eliminating their effects (as discussed above).

SUMMARY OF FINDINGS

The goal of this project was to develop an integrated system (Fig. 1, 2) for recording from single neurons in tissue culture which would complement (and in some instances perhaps replace) the Draize toxicological assay with the eventual goal of supporting a commercially viable system (see below, Commercial Market Assessment). The system consists of a number of components:

Component 1: Hardware Integration. Most of the equipment was commercially available. *Microscopic system* was found to require four stages of magnification (40-400X). The low magnification being necessary for alignment of microelectrode within the petri dish and 400X necessary to visualize the micropipette as it approached the neuron to be recorded from. Phase optics were of value in both visualizing the cell bodies and producing high quality photographs. Preliminary observation suggested the new Hoffman technology may improve visualization of the micropipette as it approached the cell. The microscope's base plate required modification to give maximum exposure of the petri dish while rigidly holding the petri dish to prevent mechanical movement. *Micropipettes* were more reproducible and had finer tips when produced with a horizontal puller than with vertical pullers. In order to provide adequate mechanical stability, the *micromanipulator* system was found to require remote control for the final penetration of the cell (the Z-axis coordinate). Mechanical hysteresis was found to be a problem in some types of manipulators and drift was a limitation of the fluid microdrive that was tested.

Component 2: Data Sampling and Analysis Software. The data acquisition package evaluated during this proposal was found to be inadequate for inclusion in a commercial system. Other data acquisition systems will be evaluated in phase II. Programs were written to sample voltage and current and to analyze the data waveforms. This system was designed to be entered into a commercial spreadsheet for summary and analysis.

Component 3: Tissue Culture. Protocols for epithelial (corneal and conjunctival) and neuronal cultures were tested and improved. Improvements include more precise dissection of the neuronal cell bodies and immediate cooling of the epithelial tissue following dissection. Matrigel and laminen were compared with collagen as a substrate but were not found to have significant advantages. To control the area in which cells were cultured, different sizes of *templates* were constructed of teflon and placed in the petri dish before plating the tissue culture cells. Scratch marks on the collagen surface were found to direct the growth of axon sprouting. Initial experiments on fetal rats were extended to newborn animals in which it was easier to differentiate the trigeminal cell bodies. In addition, cultures of

D P

C K

O

A

S

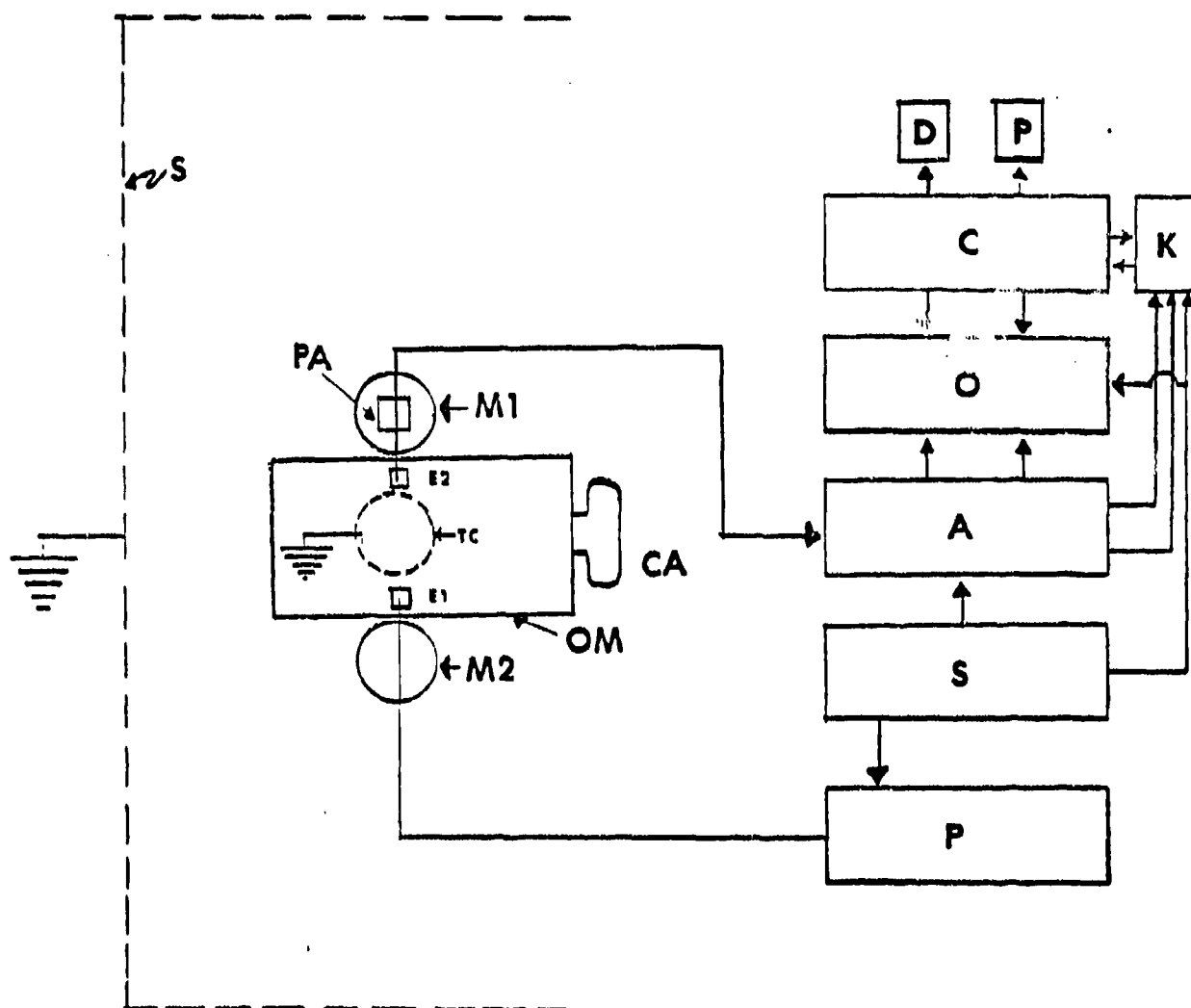P

PA ←M1

E2

TC CA

E1 OM

←M2

S

Fig. 1. Diagrammatic illustration of the experimental setup (Fig. 2) developed during this phase I feasibility study. The tissue culture (TC) preparation was placed in a specialized base plate inserted into an Olympus inverted (OM) microscope (Fig. 3) to which a camera (CA) was attached for obtaining photographic records. A specialized ground wire was lowered into the TC bath to maintain electrical connectivity (Fig. 4). A micropipette was placed in an electrical half cell (E2) and then carefully lowered into the solution using a micromanipulator system (M1) connected to a remote fluid drive. The half cell was connected to a preamplifier (PA) which is attached to the amplifier (A) for intracellular nerve recording with which the intercellular potential could be measured simultaneously with the stimulating current (Fig. 16, 17). These two waveforms were displayed on the oscilloscope (O) and sampled on a Keithley data acquisition system (K) and stored on an IBM compatible computer system with printer (P) and floppy disk (D) storage. The electrical stimulus was generated by a dual channel stimulator (S) along with a sync pulse to begin the computer sampling and the oscilloscope wave trace. The micro pressure injection system (P) used a micropipette was filled with the stimulus chemical and attached to a specialized half cell (E1). The pipette was then positioned near the cell under microscopic observation with a second micromanipulator (M2). The pressure injection system was driven by pulses from the stimulator. A wire cage (S) was also installed around the recording setup to isolate the electrical noise from the high impedance recording system. The microscope was placed on a heavy metal plate using air suspension to dampen vibration. For a detailed description of the system components see text.

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Fig. 2 Photograph of the experimental setup showing the microscope on
the left (see Fig. 3 for detailed view of microscopic setup) and the rack
containing the items of equipment on the right. Each item of equipment has a
letter label attached. From the top of the rack down, there is the Keithley
data acquisition system (G), isolation unit (F), oscilloscope (A), amplifier
system (B), electrical stimulator (C), analog tape recorder for backup of data
(D), and pressure microinjection system (E).

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adult animals were successful. The possibility of using adult animals expands the types of experiments that can be accomplished with this model system (see above).

Component 4: Intracellular Recording. After 10-14 days in culture the rat sensory neurons were found to have a normal range of resting potentials. Some neurons exhibited action potentials which likely contained a sodium activated calcium channel. After taking care to maximize sterility, it was possible to record from the preparation on different days. In some cases it was possible to record from the same neuron on subsequent days, suggesting the possibility of following the changes in a group of cells during chronic toxic exposure and/or chronic treatment for toxic exposure.

Component 5: Treatment With Environmental Pollutant. After communication with Air Force personnel, an environmental pollutant was selected and administered to neurons during intracellular recording. The neuron's responses to hyperpolarizing and depolarizing electrical stimuli were observed before and after the treatment with the pollutant; hence, demonstrating the feasibility of this model system to accomplish the goals outlined in phase I.

GENERAL METHODS

The experimental setup described below was used through a subcontract with the Department of Physiology of the University of Utah Medical Center, as administered through the University of Utah Experiment Station. Data were taken to Topical Testing laboratories for analysis. It was a major advantage to use the University facilities because of the variety of equipment available which could be evaluated for development of an optimal system.

Component 1: Hardware. The *in vitro* recording system had a number of subsystems.

Recording System. Neurons were impaled with a fine tipped microelectrode filled with KCL (3M) solution. The solution made electrical contact through a silver/silver chloride interface which was configured either as a long wire which is inserted into the electrode or as a pellet which is incorporated into the half cell (E.W. Wright, EH-1MS) that held the microelectrode. In either case, the half cell was then attached to a preamplifier through a banana-type connector. The amplifier system was a high impedance, preamplifier/amplifier system (WPI model M4-A Microprobe System, input impedance $10^{10}\Omega$). The current and voltage outputs from the amplifier were then fed to the oscilloscope for display (Tektronics, D11 storage) as well as to the computer for sampling (see above). The tip potentials on the electrode and any other offset potentials were compensated for with an offset in the amplifier. Electrode impedance was matched through bridge circuitry and capacitive compensation was used to minimize stimulation artifacts. A dual channel stimulator (Ortec 4710) generated sync voltages to the oscilloscope and computer as well as stimulus current. The stimulus current was fed through an electrical isolation unit (Grass, ISU-5) which was switched between positive to negative polarities to generate hyperpolarizing (negative) and depolarizing (positive) currents. The sync pulse from the stimulator was fed to the computer to start the data

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sampling run. Typically the duration of the hyperpolarizing or depolarizing pulses was 50 msec and began 10 msec following the sync pulse. The second channel of the stimulator was used to drive a pressure injection system (see below).

Micromanipulators. Two manipulators were necessary, one to position of the recording pipette and the other to position the microinjection pipette (see below, Results Obtained section for a discussion). The micromanipulators were secured to magnetic bases which was attached to a heavy iron base plate. To minimize vibration, the base plate was suspended using small intertubes. For heavy plates, tennis balls were also effective. The setup was located on the basement level of a relatively vibration free building. For environments with higher levels of vibration, it may be necessary to use a vibration damping table. However, for impaling neurons it was essential to have a remote drive (Z-axis) to minimize mechanical movement that occurred at the electrode tip while the electrode was being advanced toward the cell. As an improvement, a remote fluid drive (Trent Wells) was able to produce smooth, reproducible movements by changing the fluid from aqueous to a brake fluid (DOT3).

Microelectrodes. Initial experiments used a vertical puller (David Kopf Instruments, models 700C and 700B) to obtain microelectrodes relatively high impedance using capillary filament glass ($15-35$ MΩ). A Sutter Instruments (Flaming Brown, model P-87) horizontal puller was evaluated. It had precise control over several parameters of the pull including filament temperature, the velocity and timing of pull, and the number of pull repetitions.

Microscope system. An Olympus inverted microscope (Fig. 3) was used in these experiments (Olympus IMT-2). The Olympus system had a camera port in the front of the microscope to which an Olympus camera was attached for photography.

Pressure Injection System. The pressure injection system (built by the shop personnel of Topical Testing) had two channels. Pressurized gas (100% nitrogen, compressed) was passed through a regulator and fed directly to a solenoid valve which controlled the high pressure system. For the low pressure system, the high pressure line was fed to a second regulator which could vary the pressure from 0-20 psi. Thus, there was a high and low pressure range on each of the two channels. For the experiments described below, the solenoid value in the pressure line was controlled by an electrical stimulator (Ortec 4710) using 200 ms pulses at 4-5 psi with a 1 Hz repetition rate. The pressure line from the injection system was fed to the side port of a specialized micropipette half cell (E.W. Wright, model EH-2MS). The micropipette was then filled with solution and positioned under microscopic observation (200-400X) close to the cell being recorded.

Component 2: Data Acquisition and Analysis Software. The current experimental setup as configured during this phase I grant application consisted of an IBM compatible computer (808286, 16 MHz, 40 megabyte hard drive) attached to a Keithley Data Acquisition System (model 570, 12 bit A/D converter) which sampled the data from the two channels on the oscilloscope,

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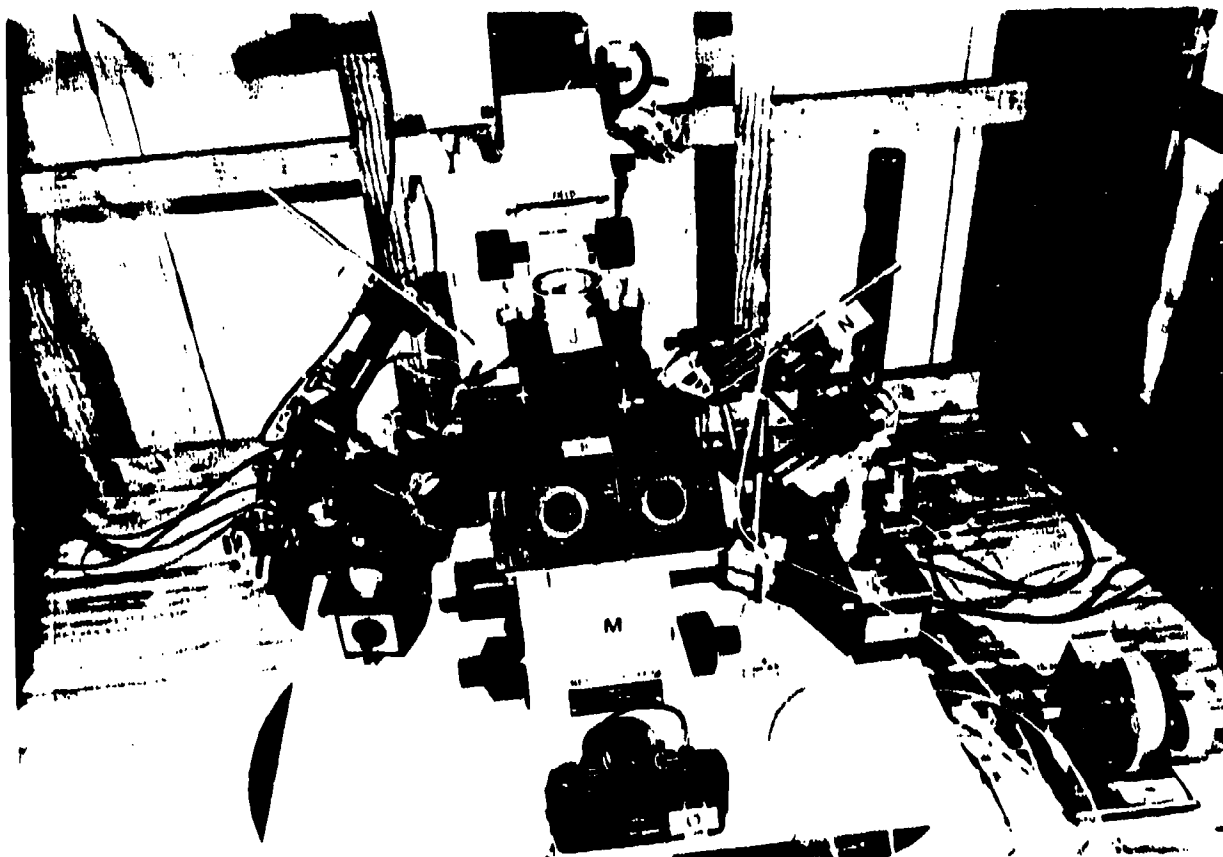
Fig. 3 Shows the inverted microscope (M) resting on a heavy steel plate and supported by miniature inner tubes. Micromanipulators (I & N) are attached with magnetic bases. The manipulator on the left holds the microinjection pipette while the one on the right (N) holds the fluid drive and the half cell which holds the recording pipette. The fluid drive is connected to a remote control on the right (L) which is resting on the table, off the base plate, so as to minimize any mechanical disturbance during positioning of the micropipette. The housing (from Hoffman) for the condenser lenses (J) is of minimal size and hence maximizes the room for positioning the micropipettes. The specialized petri dish holder (K) is shown in more detail in Fig. 4. A camera (O) attached to the microscope system for photographic records.

one channel being the intracellular voltage recorded with the micropipette and the other being the current (either hyperpolarizing or depolarizing) injected into the cell through the microelectrode. A program listing is given in the Appendix of the Final Report.

Component 3: Tissue Culture. Rats were used as an experimental model to obtain neural and epithelial cultures. Rabbits were used in a few experiments.

Establishment of Neural Cultures. Tissue from fetal rats were observed at 16-21 days gestation. The pregnant animals were anesthetized with pentobarbital (i.p.) and the fetuses were removed through an abdominal incision and euthanized via cervical dislocation. The fetuses appeared to be anesthetized along with the mother. A midline incision was made in the skull and the central nervous system dorsal to the trigeminal ganglion displaced with a rounded probe under microscopic observation (Wild dissection scope, model M5, 10-25X). The ganglion was isolated with blunt dissection, gently freed from surrounding connective tissue, cut using microscissors and placed into sterile tissue culture media. Standard tissue culture sterile technique was followed. For example, surgical instruments were autoclaved before use and dissection was done under a sterile hood. Animals were dipped in alcohol after euthanization to maintain sterility and the laminar flow hood was sterilized with UV light for 20 minutes before use.

Trigeminal ganglia were extracted from 18-21 day Spraque Dawley rat embryos after euthanization of the mother with on overdose of pentobarbital. The ganglia from 10-15 rat embryos were removed under sterile conditions, pooled in Ca^{+2}/Mg^{+2} free Hanks Balanced Salt Solution (HBSS) and gently teased apart with fine tipped forceps. The tissue was then transferred to 2 ml of 0.25% trypsin in Ca^{+2}/Mg^{+2} free HBSS and incubated at 37°C for 40-45 min. After incubation, the tissue was centrifuged at 300 g for 10 min and the trypsin solution poured off. The tissue was washed once with DMEM 10/10, recentrifuged and resuspended in 2.5 ml of DMEM 10/10 with 1 ug/ml of 7S nerve growth factor (Collaborative Research). The tissue was dissociated into a cell suspension by gentle trituration with a small diameter, fire polished Pasteur pipet. Aliquots of these dissociated nerve cells was seeded onto tissue culture plates. After incubation of the cultures for about 24-25 hrs at 37°C in a humidified 5% CO_2 atmosphere, the neurons attached to the surface of the collagen and the background cells began to proliferate. At this time, the chamber was sometimes treated with the antimitotic agent, cytosine arabinoside (10 μM in HDMEM), to reduce the background of non-neuronal cells. This treatment continued for 24 hrs, after which the neuronal cultures were maintained in HDMEM for the remainder of the culture period. Within 7 to 10 days the nerve cells generated neuritic processes.

Isolation and Culture of Corneal Epithelium. Eyes were dissected from Spraque-Dawley rats (250-500 gms) which had been euthanized with pentobarbital overdose and placed in Hanks Balanced Salt Solution (HBSS) with 500 ug/ml Gentamicin as soon as possible after dissection. Under sterile conditions, the dissection of the cornea along the boarder of the limbus was visualized with a dissecting microscope (Wild, MSA 12-15X) with care to maintain all cell layers intact and to avoid contamination with cells from the conjunctiva or

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iris. Corneas were rinsed three times in HBSS, cut into approximately 8-12 pieces and then incubated for 45 minutes at room temperature in Dispase II (1.0 U/ml in Ca^{+2} and Mg^{+2} free HBSS), a bacterial neutral protease (Boehringer-Mannheim Laboratories, Inc.). While in the Dispase solution, pieces of epithelium were peeled from the stroma with fine forceps and placed in cornea culture media (Chan & Hascke, 1982).

Dissociated cells were placed in 35mm Falcon or Corning culture plates which had been prewet with a small amount of culture media. The culture media consisted of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and either Waymouth's MB 752/1 or Ham's F12 (Gibco) supplemented with 5% fetal bovine serum (KC Biological, Inc.), 10 ug/ml insulin, 1 ug/ml hydrocortisone, 50 ug/ml Genamicin (all from Sigma) and buffered with 15 mM HEPES. In later experiments, keratinocyte growth medium was substituted (Clonetics: epidermal growth factor, insulin, hydrocortisone, Gentamicin, Amphoterin, calcium). The plates were incubated 30 minutes at room temperature with care taken to avoid dehydration. Subsequently, 0.5 ml of culture media was added dropwise to each plate. The cultures were subsequently fed twice weekly with fresh culture media and monitored regularly by phase microscopy (Forbes & coworkers, 1984).

Isolation and Culture of Conjunctival Epithelium. The eye ball was removed as described above and placed in CMFS. Under a dissecting microscope, the whole sheet of conjunctival epithelial tissue, was isolated from the limbus. The whole conjunctival sheet was rinsed three times with CMFS, then secured onto a sterile paraffin-layered 60 mm dish plate and subjected to Dispase II (1.0 U/ml) treatment at 37°C for 3 hrs in an incubator under 5% CO_2 and 95% air. The conjunctival epithelial cells were dispersed from the surface by gentle pipetting several times with CMFS. Cells were finally collected by centrifugation at 800 g for 5 min. (Tsai and Tseng, 1988).

Tissue Culture Templates. Tissue culture chambers were prepared according to the method of Ziegler and Herman. After coating 35 mm Falcon or Corning tissue culture plates with 0.5 mg/ml calfskin collagen (Vitrogen 100, Collagen Corp., Palo Alto, CA), a series of 20 parallel scratches about 360 um apart were made in the collagen coating and the superficial surface of the culture plate. These scratches were made using a rake consisting of (20 insect pins cemented together) and then pulling across the collagen surface in one even stroke. An 8 mm glass cloning cylinder (Bellco Glass, Inc., Vineland, NJ), with one end covered by a fine ribbon of high vacuum silicone grease (Dow Corning, Midland, MI), was secured to the collagen surface with gentle pressure. In other cases, the teflon templates of different sizes and shapes were evaluated (Fig 5). If the level of medium in the inner chamber dropped significantly prior to plating the nerve cells (indicating a leaky seal), the culture plate was discarded (Forbes & coworkers, 1987).

RESULTS OBTAINED

The purpose of this phase I study was to determine of the feasibility of using an *in vitro* model to approximate the peripheral nervous system component of eye irritation tests which have traditionally been done on awake animals. The tissue culture system was complex in that it contained a number of

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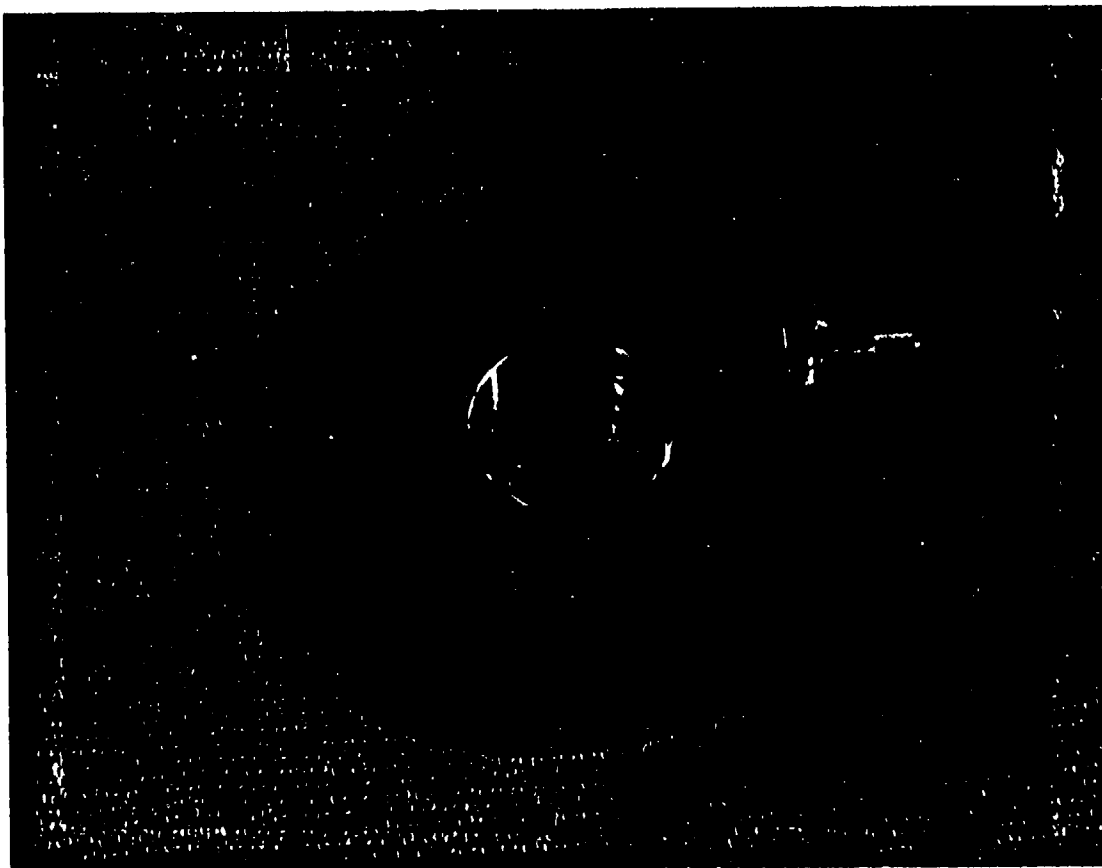Fig. 4 Shows a specialized base plate for the inverted microscope (Fig. 3). The plate is made of machined plastic and holds the petri dish securely. Note that the entire bottom of the petri dish is visible. On the top of the plate is a clamp which is machined to hold the ground wire (which is removable and made of silver with a silver/silver chloride coating). This specialized assembly was made by Topical Testing shop facilities.

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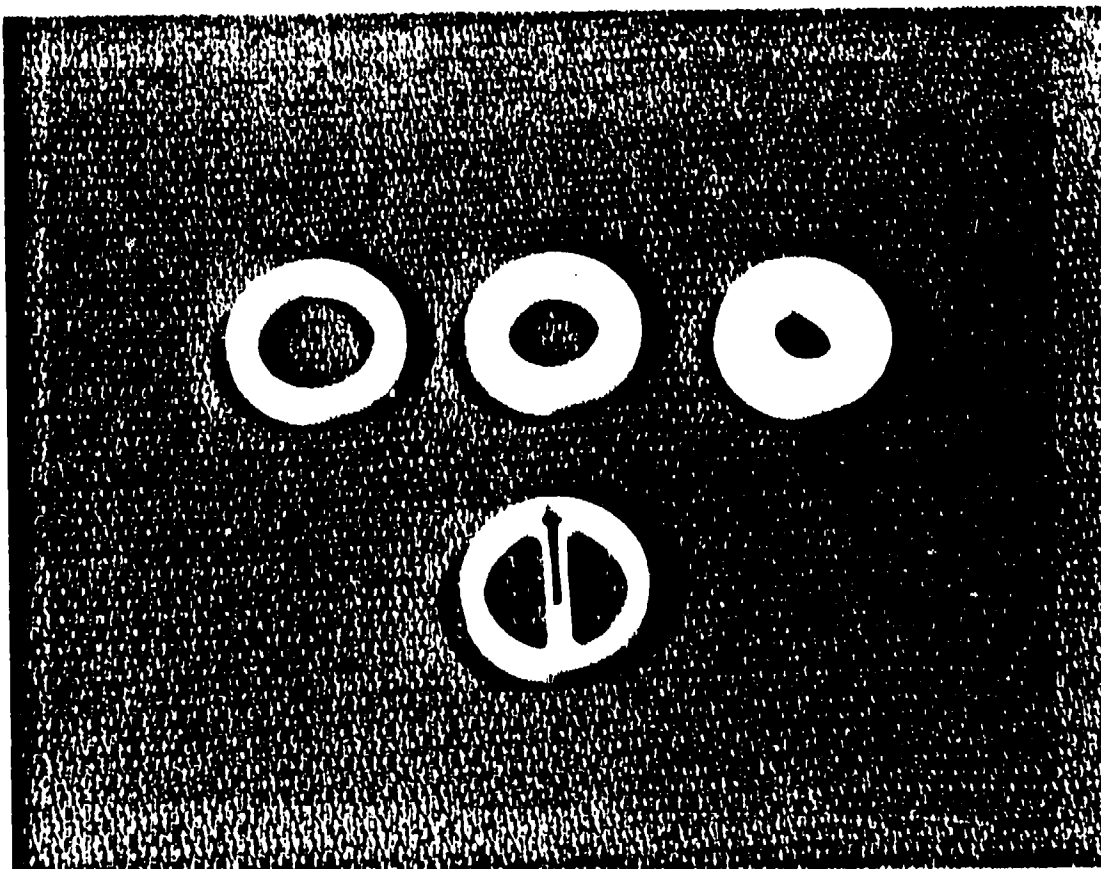
Fig. 5   Shows various sizes of templates used to limit the growth of the
tissue culture in the petri dish.   The templates were made of teflon and
machined to precisely fit within the petri dish.   Sterilized silicon grease
is used to seal the chamber to the bottom of the dish and prevent leakage of
cells and media.   On top are shown the different sizes of circular templates
used to study the effect of cell density on neuron and target cell growth and
function (see Task 9 in phase II proposal).   The bottom template is keyhole
shaped in which the cells are plated into the elongated area.   The round area
at the top of the keyhole structure is used to feed the cells.   Hence, this
template has been used to study both guided axon growth and the effect of
having a high density of neurons plated in a small area.

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Fig. 6 Shows a picture of the injection pipette at 200X view. The pipette was filled with fast red dye to mark the area injected. The injection pressure was 5 psi, duration approximately 500 ms.

functional components. One objective of this proposal was to determine whether these components could be brought together into a system which was viable for phase III commercial development.

Component 1: Hardware Integration. There were a number of pieces of equipment necessary to perform the required job as described above. These included the neural recording, pressure injection, data acquisition, and the microscopic systems. The evolution of each system is discussed below.

Microscopic System. The microscope's (Fig. 3) base plate had a circular hole in the center to pass light from the objectives lens through the preparation. The petri dish was placed over the hole and the base plate moved manually using X- and Y-position controls to observe the tissue. This base plate system was found to have major disadvantages. The hole in the plate was not big enough for the whole petri dish to be observed, the petri dish was not secured to the plate and the ground wire for electrical recording was not attached the base plate. Hence, a new plate was fabricated by the shop personnel (Fig. 4) which was made of plastic and was thick enough that the petri dish could be securely mounted inside the hole and was machined such that the entire base of the dish could be viewed through the microscope. In addition, a ground-wire holder was mounted to the base plate which allowed the ground wire to be easily raised and lowered into the tissue culture solution. An additional advantage was that by having the ground-wire attached to the base plate, it was possible to rotate the whole preparation without disturbing the ground and hence find an optimal orientation for aligning the microelectrode with the neuron to be studied.

Initially the microscope was fit with a 10X bright field objective and 20X and 40X phase objectives. The 20X and 40X phase objectives were ultralong working distance in order to focus through the petri dish. Adjustable rings were beneficial in that the working distance of all objectives could be adjusted so that the focal plane remained the same for all magnifications. Hence it was possible to efficiently switch from one power to another without refocusing. In addition, it was found that phase produced a clearer image than bright field. Thus, all objectives were eventually changed to phase contrast. In order to impale a neuron, the microelectrode was lowered into the solution and then gradually and carefully brought into alignment with the neuron to be studied. When the electrode first entered the solution, it was important for the experimenter to see the electrode's location in the bath and hence, position it accurately. It was found that the 10X objective had too small a field to locate the electrode when it first entered the tissue culture bath. Hence, it was necessary to install a lower power objective (4X phase).

The microscope was also equipped with Hoffman objectives (20X and 40X) which enabled viewing of the cell surface (Figs. 8, 10, 12). The phase and Hoffman condenser lenses were fitted on a slide condenser system obtained from Hoffman which allowed two condensers to be mounted on a slider mechanism. To change condensers, one slider was pulled out from the front of the microscope and the other slider inserted. This slider system had the great advantage that it was narrow (took up very little room) and hence presented a minimal interference with positioning the microelectrodes.

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The microscope's power supply was found to generate electrical noise which had to be screened with grounded metal plates and foil. It is recommended that a DC power supply be implemented. It was also found useful to place a grounded cage over the setup to eliminate electrical interference from the surrounding environment.

Microelectrodes. Two types of micropipette pullers were evaluated. The vertical pullers (Narishige) were found to be inadequate for recording in tissue culture in that the pipette produced mechanical distortion of the neuron before penetration of the cell membrane, often resulting injury to the sensory cell body as indicated by a low, unstable resting membrane potential and short recording time. Cells were difficult to penetrate and in some cases cells lost their attachment with the collagen sublayer. In contrast, the horizontal puller (Sutter Instruments) produced sharp tipped pipettes of high impedance which often allowed penetration of cells without noticeable injury. It was necessary to silver/silver chloride both the ground wire which had been installed in the system and the microelectrode half cell (holder) which contained either a silver wire or a silver pellet inside the half cell.

With some experimentation it was possible to obtain the electrodes with a very fine taper and impedances ranging from 60-120 MΩ with the horizontal puller. These electrodes had the advantage that they seemed to be very smooth and sharp and hence were able to impale cells with little damage. Hence, recording stability increased substantially after switching to the Sutter puller. The puller had a digital memory which allowed storage of several different stimulus protocols. Thus, it was possible to switch protocols and produce either recording pipettes or pressure-injection pipettes with a high degree of reproducibility and accuracy. The pressure-injection micropipette had about a 5-7 μ tip (1-3 MΩ resistance when filled with (3M KCl) and no capillary filament (1.5 mm, o.d., A-M Systems #50328). It was found that by using this diameter efficient injections could be obtained while the tip was small enough that the solution did not diffuse out of the electrode prior to the pressure injection.

Micromanipulators. Two Narishige motorized, mechanical drive micromanipulators with remote control were evaluated. One (model SM-21) was not of proper design for tissue culture in that it was heavy and its X and Y-axes were difficult to adjust. In addition, the Z axis was rather course for these experiments. The second Narishige (model ME-71) was adequate for cell penetration; however, it was an older model and was not linearly controllable over much of its range. A third model tested was a Narishige manipulator without manual mechanical remote control (model MP-2). It was found that without remote control there was too much random movement of the micropipette tip for the adequate cell penetration. To improve this system, a fluid drive with remote control (Trend Well) was mounted to the manual manipulator. The fluid drive was found to be more effective when filled with brake fluid (DOT3) rather than the aqueous solution. However, the fluid drive manipulator also had a problem of mechanical hysteresis in that it would track linearly when approaching a cell but would scrape on the collagen substrate on retraction (see below). However, this configuration was adequate for cell penetration and was used for the recordings described below. A preliminary evaluation of the Huxley style manipulator showed to be relatively low in cost ($2,500-

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3,000) and to provide excellent mechanical stability. However, our evaluations show that it also contains mechanical hysteresis. Therefore, recommendations for phase II work includes the use of a Huxley style manipulator (e.g., WPI, Model M757) with a remote controlled, Z-axis manipulator without mechanical hysteresis such as a piezo electric microdrive.

Component 2: Data Acquisition and Analysis Software. The Keithley data acquisition system was difficult to install. For example, the one chosen for our experiments, a clone system (Burgoyne IBM clone 80286, 16MHz, IM RAM) was unable to sample at rates greater than 6 kHz total through put (although Keithley's specs showed a 33 KHz maximum sampling rate in the single ground mode). Topical Testing personnel had several conversations with Keithley but were unable to resolve the issue. Fortunately, the 6 kHz rate was adequate for the current application. The personnel of Topical Testing have had experience with the Keithley system on a number of computers (Zenith 286, IBM XT, AT&T 286) and have found that each installation required a substantial commitment of effort and time. Hence, from this preliminary observations, it was concluded that for phase II development, Topical Testing will evaluate other data acquisition systems to determine which will work on a variety of IBM compatible computer systems without substantial installation costs.

The software was written by the principal investigator in compiled BASIC (Microsoft Quickbasic Ver. 4.0). This data acquisition program operated within Quickbasic environment with the use of Keithley's Quick500 data acquisition software. A sync pulse from the stimulator was used to trigger the Keithley system to start sampling for a duration (100-200 ms) which was adjustable. The sync rate was initially set to 1 Hz. The hyperpolarizing and depolarizing DC current pulses were usually 50 msec in duration and were begun approximately 10 msec after the start of data sampling. With this stimulus paradigm it is possible to record the amount, timing and polarity of the hyperpolarizing and depolarizing current pulses as well the resulting charges in membrane voltage. Provisions were made in the software to record membrane potential following the chemical stimulus without current stimulation. The two data files for stimulation and the membrane voltage were recorded in a digital file along with an ASCII file describing the parameters of the stimulus and other variables. Hence, it was possible to load the ASCII information file and the two data files into a spreadsheet format for subsequent analysis. The listings for the programs discussed above are contained in the Final Report (Appendix).

Component 3: Tissue Culture. The culture of target tissue (corneal and conjunctival epithelium) followed standard protocols as described above. However, modifications were necessary.

Corneal Epithelium. It was found that for dissociation of corneal epithelium it was helpful to leave the epithelium in the enzymatic solution for a longer period than called for in the original protocol. Because neurons had been found to grow adequately on a collagen substrate. We therefore tried using collagen for our corneal epithelial cultures and were not obtaining great success. Therefore we began experimenting with other substrates (e.g., laminen and Matrigel). In general we observed that the epithelium that was dissociated epithelial cells tended to stick better to the substrates than did

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fragments of epithelial tissue. Increased enzymatic dissociation time enhanced cell dissociation. Eventually we were able to grow corneal epithelium in culture changes in protocol included adding epidermal growth factor to the culture medium and it appeared to be important to place the cornea immediately on ice after extraction. The Matrigel and laminen did not appear to have obvious advantages over the collagen as a substrate and, especially Matrigel seemed to have the disadvantage of producing a glare when viewed microscopically such that it was difficult to focus on the individual cells after plating. We have been able to grow the corneal epithelium for up to 3-4 weeks (Figs. 7 & 8). The epithelium grows in clusters and near the end of the growing cycle small fascicles appear in some of the cells which probably indicates that it is near the end of its growth period.

Conjunctival Cultures. The dissociated conjunctival epithelial cells were dissociated using a protocol as described above (Figs. 9 & 10). As with the corneal epithelium, the conjunctival cells were spherical in shape when first dissociated. After plating, cells began to attach to the collagen surface within 24 hours and continued to attach for several days.

Neuronal Cultures. The neuronal cultures (Figs. 11 & 12) were obtained as discussed above. The ganglia were difficult to differentiate from surrounding tissue with the younger animals (less than 16 days). By 18 days ganglion were clearly discernible. In newborn rats (1-5 days) it was possible to clearly identify the ophthalmic and major branches of the trigeminal ganglion.

The neurons appeared to grow equally well on collagen, laminen and Matrigel and hence we chose to continue using the collagen. For neuronal dissociation, the isolated tissue was placed in dissociation media in a culture tube and the tube was positioned close to horizontal in an oscillating water bath using a holder designed by the shop facilities. Gentle mixing enhanced the dissociation while lessening cell damage. An even greater improvement in dissociation was due to the more precise localization of the sensory cells in the ganglion and hence be able to trim off as much excess of the nerve fiber filaments as possible before beginning the dissociation. We speculate that in the original protocol cell bodies that were sticking to the fibrous tissue and hence being removed with the media change rather than being suspended in the aqueous solution and gradually settling onto the collagen substrate. Hence, by minimizing the fibrous tissue, it was possible to greatly enhance the yield of neurons.

At first the cells were dissociated by taking the whole ganglion plus a small length of nerve track above and below the ganglion and cutting it into smaller sections. However the yield of neurons was low using this procedure. Gradually, over the six month grant period we were able to identify the sensory neurons more accurately and with greater confidence. Initially it was difficult to determine precisely what the location of the cell bodies of the trigeminal ganglion; however with the assistance of a coworker (Dr. Alcayaga) the Principal Investigator was able to compare the sensory neurons in the in vivo rat cervical ganglion with the trigeminal preparation and learn to identify the trigeminal sensory neurons. It was possible to identify their
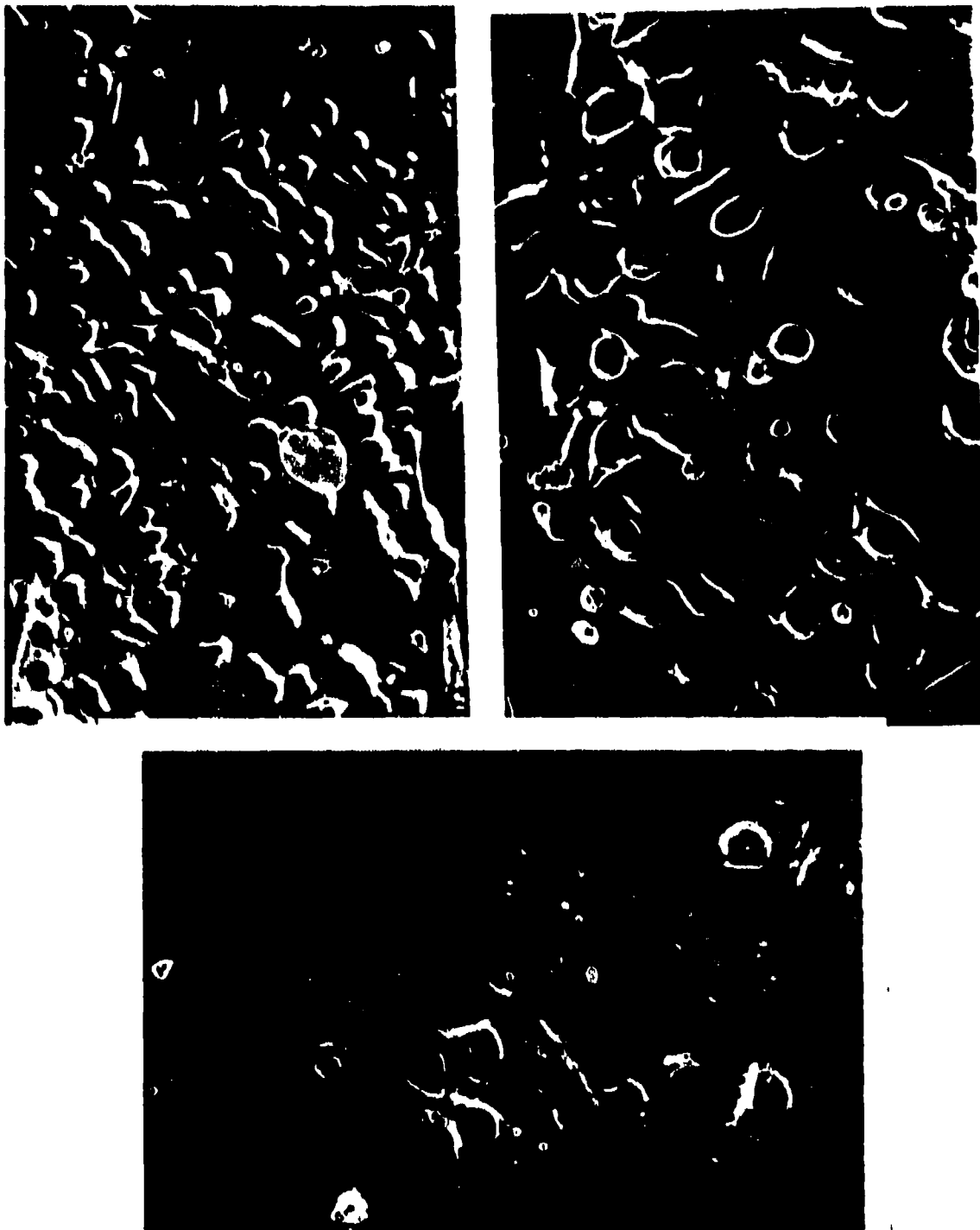
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Fig. 7 Illustration of corneal epithelium as viewed under phase microscopy. The top two photos show corneal epithelium growing towards convergence ten days after plating (left, 200X; right, 400X). The bottom panel shows "mature" corneal epithelium seven weeks after plating in co-culture (Fig. 13).
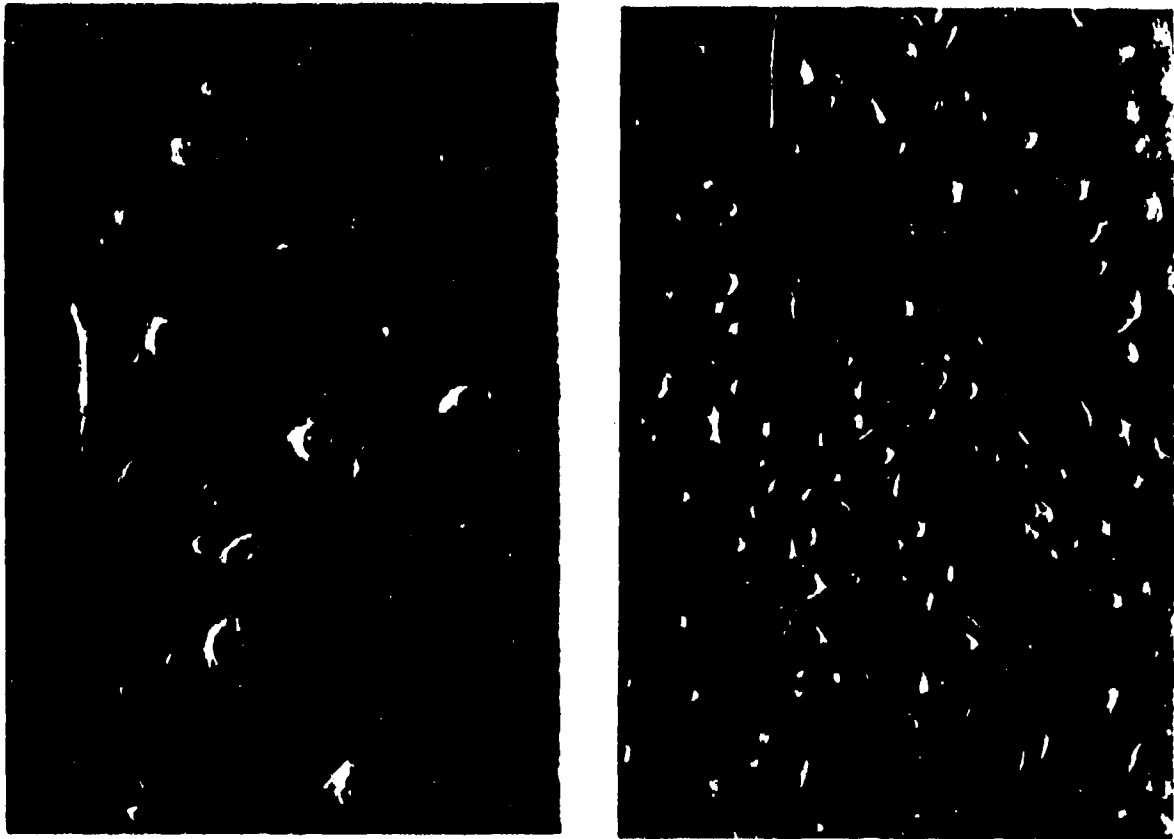
Fig. 8   Illustration of corneal epithelial cultures shown in Fig. 7 as
viewed under Hoffman optics.   (Left, 200X; right, 400X).

Fig. 9 Conjunctival epithelium at ten days (left, 400X) and seven weeks (right, 200X). Phase optics.

Fig. 10   Conjunctival epithelium after ten in days culture (left, 200X; right, 400X).   Hoffman optics.
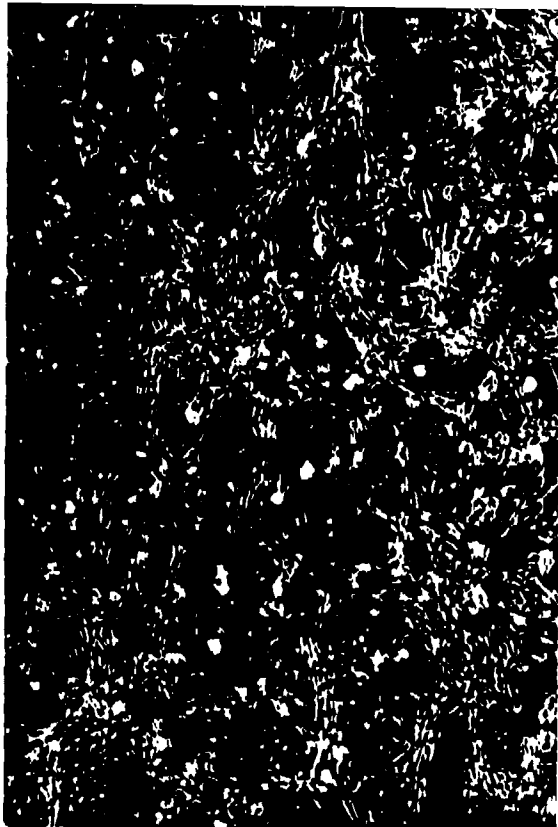
Fig. 11 Neurons in culture, three days after plating. No fibroblast
inhibitor (e.g., cytosine arabinoside) was given; hence, the neurons were
growing on a fibroblast network. The neurons are shown with phase optics
(upper left, 40X, upper right 100X, lower left 200X, lower right 400X). As
shown under the highest magnification (lower right) the neurons (arrows) are
often oval in shape and "light-up" with phase optics magnification.

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Fig. 12 A neuron viewed under Hoffman optics (400X). Note the axonal process extending off the cell body. This cell was recorded from and exhibited action potentials when depolarized, thus confirming that it was a neuron.

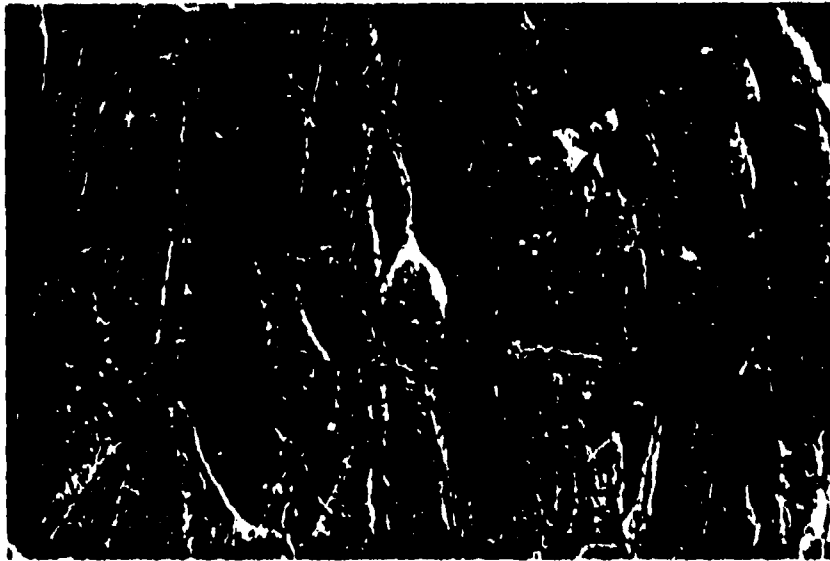appearance under the dissection scope in the animal and after the ganglion had been isolated in media at 20-50X. The ganglion cell bodies were located in small protruding areas on the dorsal surface of the ganglion and had a more distinctive vascular supply and slightly darker appearance than the surrounding ganglion tissue which contained nerve fibers and connective tissue and hence can be trimmed off the top of the ganglia after they have been isolated in culture media. It was discovered that by harvesting only the segments of ganglion containing cell bodies it was possible to greatly enhance the yield.

We experimented with different sizes of plating areas in a dish. As described above, this was accomplished by constructing templates of different sizes and shapes (Fig. 5). To limit cell migration, the template was secured to the collagen surface with sterilized silicone grease. The teflon templates were sterilized by autoclave and reused numerous times. Preliminary evidence suggested that suggest that axon sprouting may be enhanced at higher all density. For example, in cultures where a long, thin template was used (similar in shape to a keyhole), the neurons seem to form a more substantial network. Scratches in the collagen surface controlled the direction of axon sprouting such that axons tended not to cross over scratch marks and hence directed growth could be obtained. Other templates were doughnut shaped with various size holes (Fig. 5). Templates could be removed without disturbing the neural network and hence might be a valuable tool in the future to focus the growth of epithelium and neurons to a smaller area on the petri dish so that they can be efficiently studied. Generally neurons established axons in about 7-10 days and cultures have been maintained up to 10 weeks.

In addition to experiments performed on fetal and newborn animal, neurons from adult rats (250-500 gms) were successfully accomplished. The use of the dissection procedure which separated the cell bodies from fibrous tissue (see above) greatly enhanced the yield from adult animals using protocols used for fetal rats. The use of adult animals might be advantageous in some experimental designs. For example, adult animals could be exposed to environmental toxins for a control period of time and their neurons cultured. Through comparisons with unexposed rats, tissue culture could help reveal whether _in vivo_ exposure had a long-term influence on sensory nerve function. These results could then be compared to those obtained when the neurons are first exposed to the environmental chemical during tissue culture. Hence, a determination could be made if exposure to the toxins has a similar effect on the neurons whether it is _in vivo_ or _in vitro_. In addition, the exposure of the animals to a toxin _in vivo_ would be analogous to the situation in which the human work force is exposed to environmental pollutants.

A few cultures of rabbit trigeminal ganglia and corneal epithelium were obtained from newborn rabbits, using protocols similar to those described above for rat, thus demonstrating the feasibility of the phase II rat/rabbit comparisons outlined below.

Co-cultures. The corneal and conjunctival epithelium (Figs. 13, 14) began to attach to the collagen substrate within 24 hours of plating and approached confluency (depending on the number of cells plated) in about seven
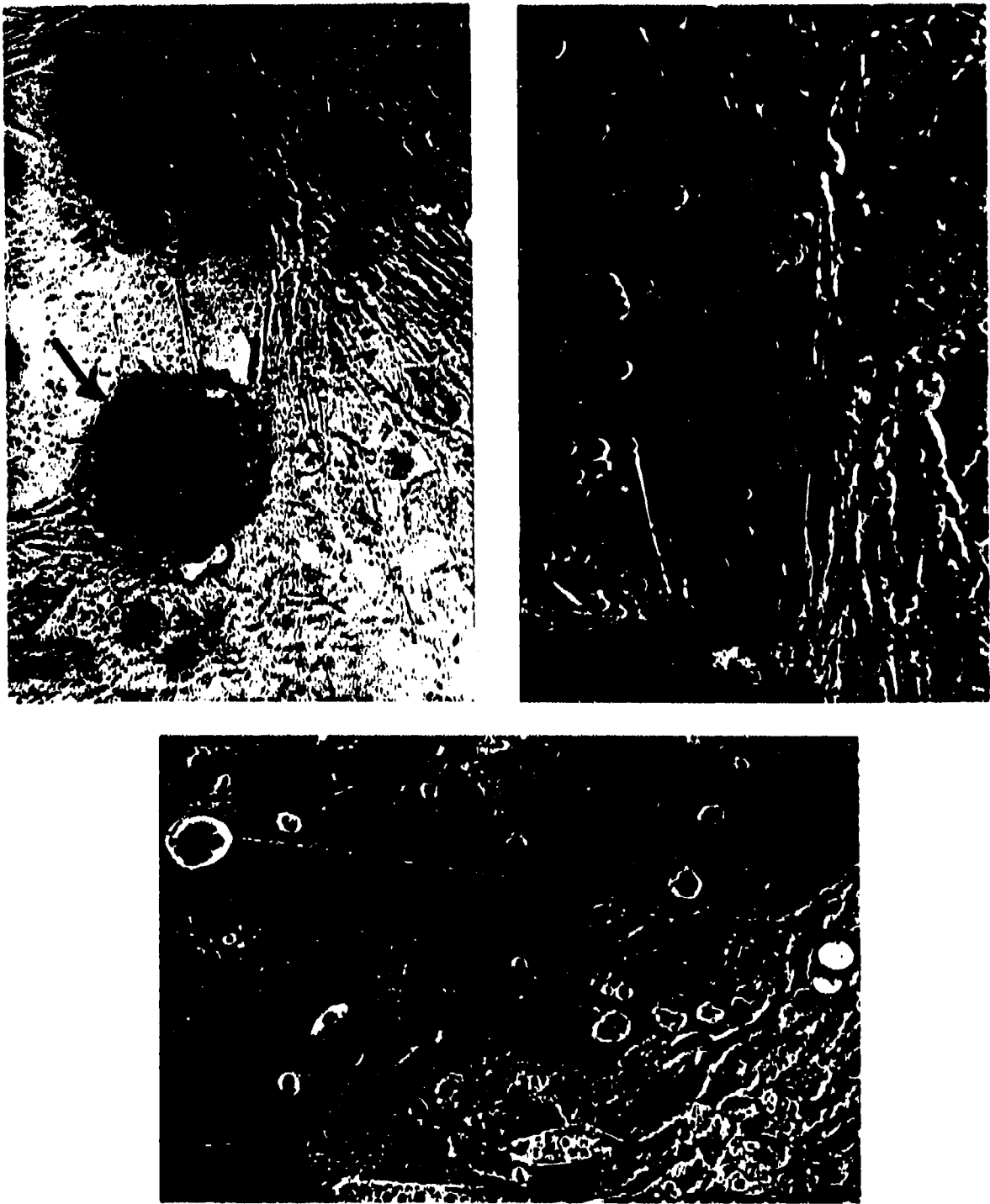
28

Fig. 13 Illustration of neurons grown in co-culture and different patterns of growth seen in different sections of the tissue culture. The epithelium was plated first (seven weeks prior to photograph). Neurons were then plated four weeks after the epithelium (two and a half weeks before the photograph was taken). The figure is divided into two parts. In Fig. 13a, the top left panel shows a clustering of nerve cells which often occured in culture. (The dark, circular area in the left lower quadrant of the photo). The upper right panel is a magnification (400X) of the upper left panel showing axonal processing extending from the mass of neurons (see arrows). The bottom photo shows an isolated axon with a neuronal process running to the right and terminating in the tissue on the right had side of the photograph (400X). Phase optics.
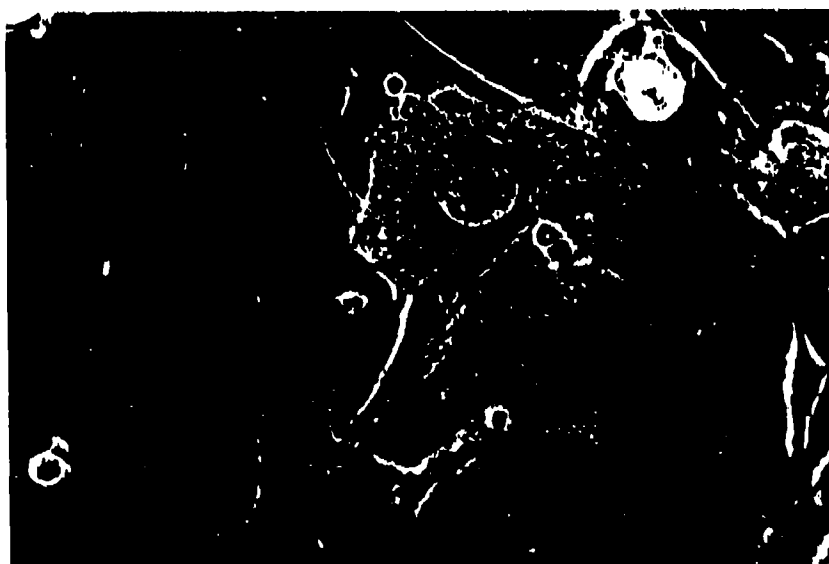
30

Fig. 13b Shows another part of the tissue culture where the neurons were growing on top of a dense population of cells (probably a mixture of fibroblast and epithelium). The neurons in the upper panel are oval shaped (arrows). The halo of light is produced by the phase optics. The lower panel shows an illustration of mature epithelium (400X). Phase optics.

days. Consequently, it is estimated that a minimum of 17 days will be required before a co-culture preparation is ready for study (7 days for the initial epithelium growth plus another approximately 10 days for neuronal growth), if the epithelial cells are allowed to "mature" before the neurons are plated. Alternatively, minimum culture time may be reduced to 10-12 days if epithelium and neurons are cultured simultaneously. Phase II experiments are designed to test both strategies.

Component 4: Intracellular Recording. After 10-14 days in culture, recordings (Fig. 14) showed neurons to have a normal range of resting potentials of up to 70 mv. Neurons generated action potentials when the membrane was depolarized to threshold levels (Fig. 15-17) and trains of action potentials at suprathreshold levels of depolarization (Fig. 16). In some neurons, action potentials exhibited a "humped" shape upon their falling phase which is likely due to calcium channel activation. There has been no evidence of delayed rectification during hyperpolarization (Fig. 17). Drift, or instability, of the DC recording was reduced by silver/silver chloriding the silver ground-wire in the microelectrode half cell. Additional stability was attained by touching the silver ground and recording surfaces together in a completed electrical circuit for several hours to equalize their surface potentials. As described elsewhere, impalements were improved by using high impedance pipettes (60-120 MΩ) and by improving the manipulator system. Neurons which had been in culture for 3-4 days were difficult to impale, possibly due either to the flat shape of the cells before they reach maturity and/or the outer surface of the neuron being too compliant to allow adequate penetration. Our initial impression was that both might be significant factors. However, after 10-14 days in culture, the impalements were obtained more readily and the axonal growth was visible. Hence, initial observations suggest that leaving the neurons in culture for about 10-14 days before testing will be adequate.

Component 5: Treatment with Environmental Pollutant. The Principal Investigator on the proposal contacted the contracting officer who authorized contact with Col. Harvey J. Clewell at Wright-Patterson AFB concerning a pollutant that would be of interest to test. The Principal Investigator, upon talking to Dr. Clewell, had further discussions with Marilyn George and Nickolas Del Raso. It was determined that nonadecafluorodecanoic acid (PFDA) was of interest. Following Mr. Del Raso's suggestion, PFDA (10mg) was predissolved dimethylsulfoxide (DMSO) (5ml) and then diluted in distilled water to a concentration of 50mg/ml. These operations were performed under a hood using standard safety procedures. Pressure injection micropipettes (see above) were then loaded with the PFDA and transported in a pipette container to the recording setup. After impaling a cell for intracellular recording, the pressure injection pipette was positioned under magnification (200-400X) close to the neuron under study. The distance from the recorded cell was determined previously by using a dye pressure injection to determine the envelope (or area) which would contain the highest concentration of expelled fluid (Figs. 6, 18). At the time of injection, the stimulator was used to drive the pressure injection circuitry and the response of the neuron (grown singly or in co-culture) to hyperpolarizing and depolarizing currents was recorded before and during pressure injection of PFDA.
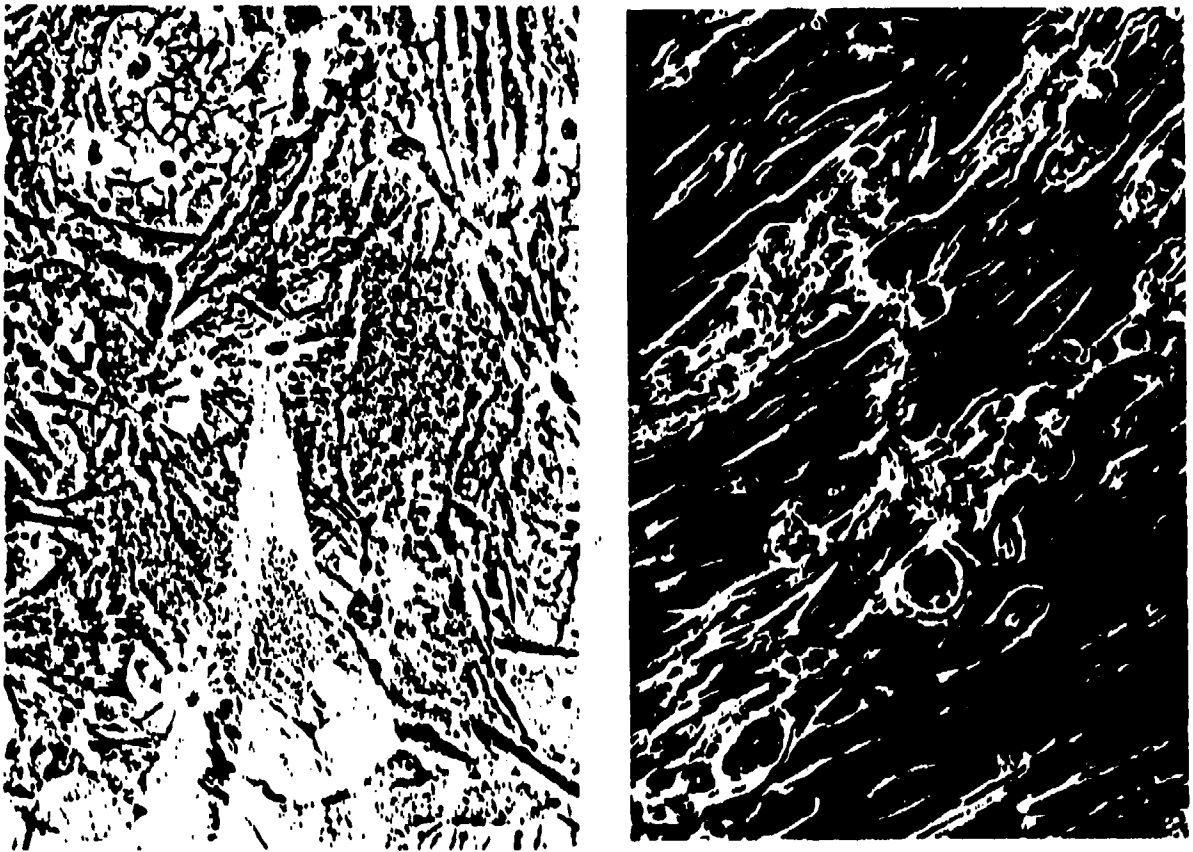
Fig. 14   Ilustrations of mic. pipettes (see arrows) impaling neuronal
cells grown in isolation (the left-hand panel, Fig. 11) and in co-culture
(right-hand pannel, Fig. 13).   Phase optics.

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Fig. 15 Illustrates typical intracellular recordings (top trace) from a
neuron to depolarizing (A) and hyperpolarizing (B) electrical current (bottom
trace). The depolarizing pulse was at threshold amplitude (1.5 picoamps [pa])
and the hyperpolarizing pulse was 0.17 pa. The numbers displayed show the
output from a computer program which measures different parameters of the axon
potential. Membrane voltage, -42 mv; action potential, 56 mv; overshoot, 13
mv. Total time - 50ms.

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Fig. 16 Illustrates the typical response of a neuron to depolarizing current of increasing amplitude. In A and B, the depolarizing stimulus was 10 ms in duration and was 0.5 and 0.71 pa, respectively. As the depolarizing current was increased in amplitude, more action potentials were recruited. The depolarizing current for D-F was 0.81, 1.47, 1.69, and 2.06 pa (50ms duration), respectively. Membrane voltage, -55 mv. Total time — 50ms.
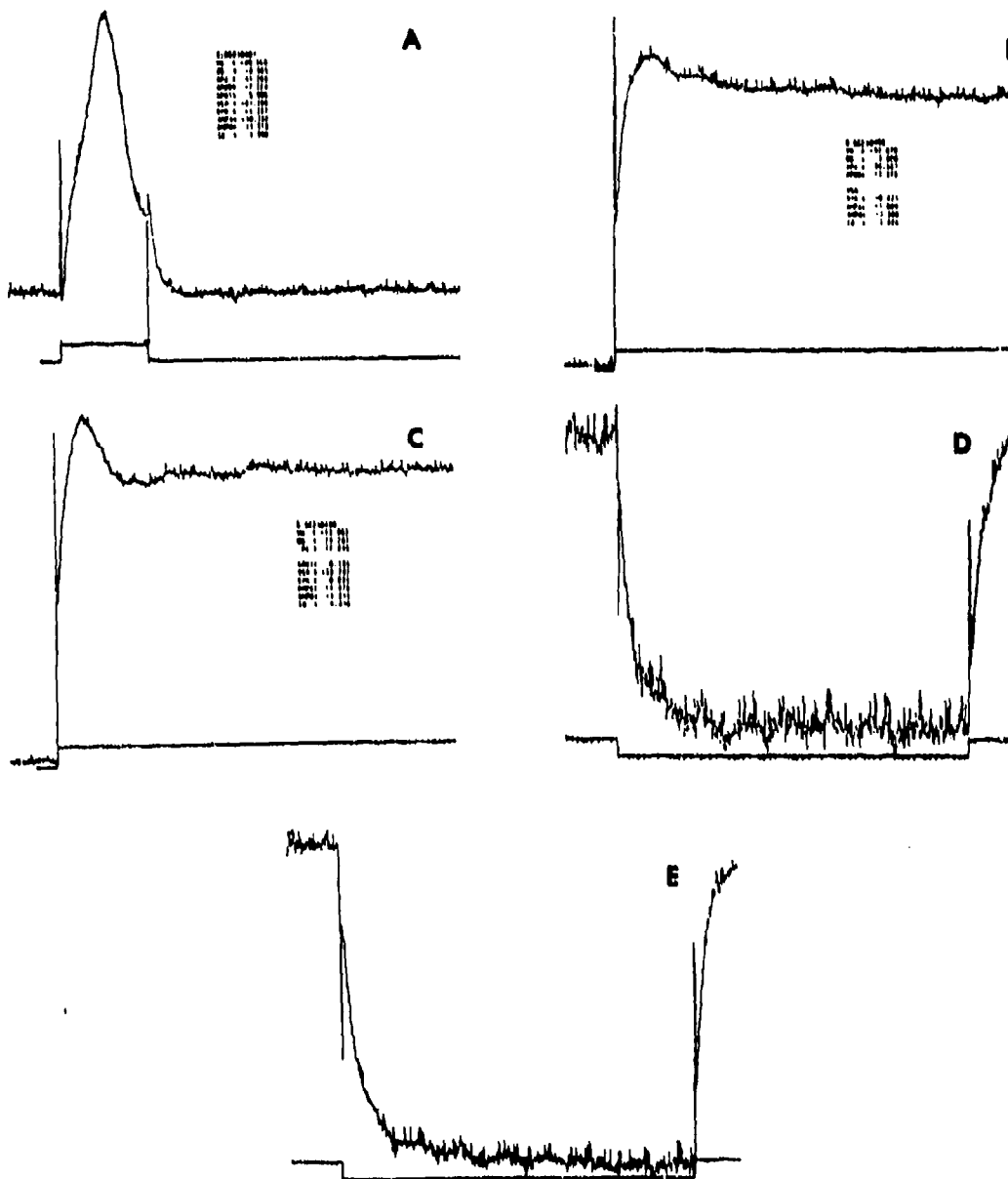
Fig. 17 Illustrates a commonly occurring event which suggested that a sodium-activated, calcium channel may be a component of action potentials in some neurons. A shows a typical action potential generated at the first of the recording by a relatively low current (1.9 pa). After hyperpolarizing stimuli were given (D and E), the sodium spike was blocked leaving a residual voltage dependence spike. [Note that unlike a sodium spike which is an all or none in nature (either none or it goes to full amplitude), the spike was voltage dependent in that action potential amplitude increased as the current was increased.] For example, in B the action potential was smaller than in C and the current was 3.3 pa in B versus 4.3 pa in C. D and E illustrate the response to a hyperpolarizing pulse which was 0.78 pa in D and 0.13 pa in E. There was no evidence of delayed rectification in this neuron, which was typical for the neurons sampled thus far. Membrane voltage, -52 mv. Total time - 50ms.

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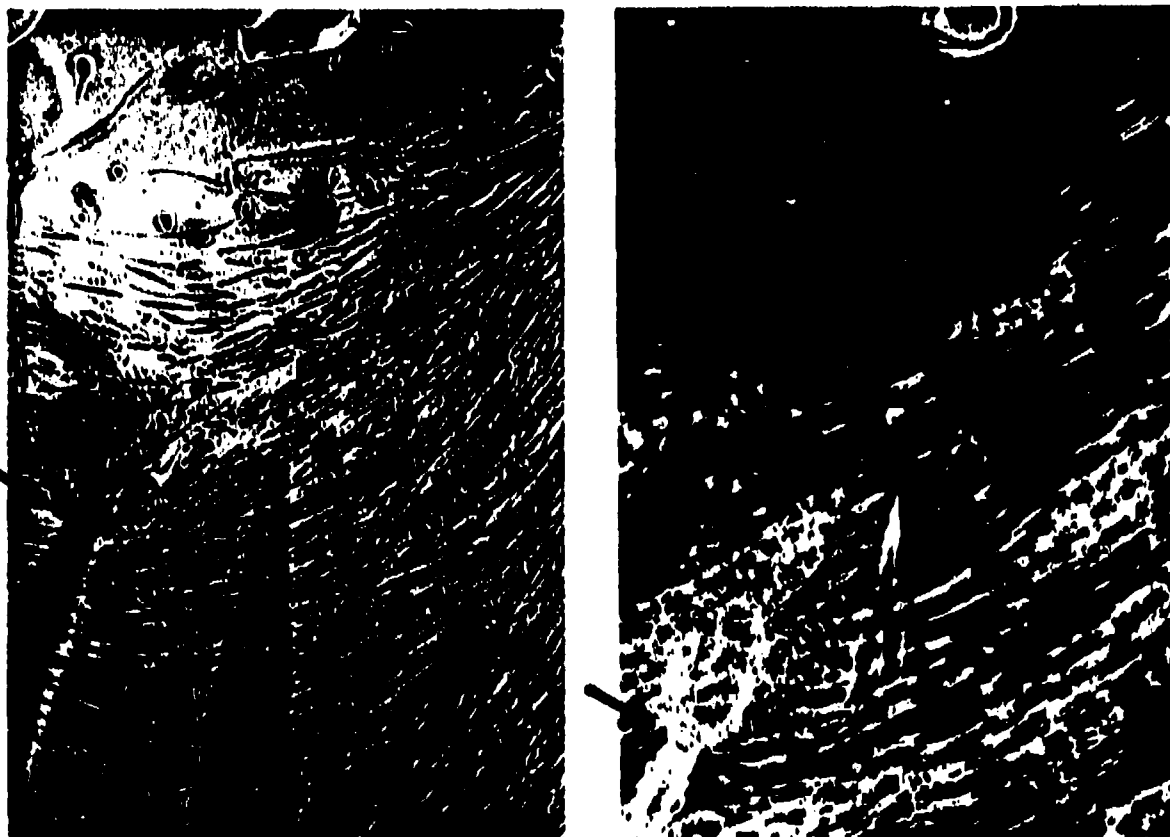
Fig. 18 Illustrates the positioning (see arrows) of microinjection and
recording pipettes. The left-hand figure shows the silhouettes of the
injection pipette (left margin of photo) and the recording pipette (center of
the photo) under 200X magnification. The right photo shows the injection
pipette (lower left-hand corner) and the recording pipette (center of the
photo) at 400X magnification. Note there was some fluid leaking from the
ejection pipette indicating that these pipettes should have a smaller tip
diameter to prevent passive diffusion of the chemical into the cellular
environment. Phase optics.

So far the neurons have not displayed a response to the chemical stimulus. There are at least four reasons why this might have occurred: 1) the neurons were too young to have a normal response to irritants (5-10 days in culture from newborn animals), 2) the concentration of pollutant may have been too low in that it might be binding to protein in the solution, 3) the neurons may require a substantial innervation with target tissue before they display a response to the environmental polluting agent, or 4) due to micromanipulator instability, the neurons might require a longer exposure time to the chemical stimulus than these feasibility experiments allowed (60-120 sec).

RECOMMENDATIONS FOR FURTHER DEVELOPMENT

The phase I feasibility study has demonstrated that the total package for growth and recording of neurons in co-culture with epithelium as well as the micro exposure of these neurons in tissue culture to environmental pollutants, is a viable model. All components of this system have been tested and are operational. A phase II grant application has been submitted which defines a set of tasks which will lead to a commercially viable system complete with the hardware and software components as well as a standardized data base of tissue culture responses to a chosen set of environmental pollutants. See the phase II proposal for further details.

APPENDIX

The appendix contains computer program listings for software discussed above.

```basic
'filename: adc3.bas :'11/27/89
'hit F5 (NOT shift-F5) to run program

d1$ = "trigeminal"
d2$ = "coculture (Y/N)": d2a$ = " "
d3$ = "type of coculture ..": d3a$ = ""
d4$ = "from..": d4a$ = "rat"
d5$ = "from .. day old": d5a$ = ""
d6$ = "from ..weight": d6a$ = ""
d7$ = "recording .. days after culture": d7a$ = ""
d8$ = "coculture was .. days old": d8a$ = ""
d9$ = "chemical was ..": d9a$ = ""
d10$ = "injected at time ...": d10a$ = ""
d11$ = "chemical conc was .. ": d11a$ = ""
d12$ = "chemical vol was approx ..": d12a$ = ""
d13$ = "template design was ...": d13a$ = ""
d14$ = "initial neuron conc was ..": d14a$ = ""


stimdur = .1: 'stimulus duration was 100 ms

DEBUG = 0
'A/D range set to +-10 volts
'expected max voltage range of input signal is +-.250 volts (250 mv)
'current=volts/20 Mohm= volts*(0.05*10**-6) OR volts*50*10**-9; therefore:
'      current in nanoamp=volts*50
'            therefore:volts range of +-.250 v is current range of +-12.5 na

'A/Dunits min= 0,   A/Dunits max= 4096
'A/Dvolts min= -10, A/Dvolts max= +10
adunitmin = 0: adunitmax = 4096
advoltmin = -10: advoltmax = 10
'membrane volts min= -250mv,     membrane volts max= +250mv
'membrane current min= -12.5na, membrane current max= +12.5na
'voltratio= /(A/Dmax(=4096)-A/Dmin(=0))/(memvoltmax(=.25)-memvoltmin(=-.25))
voltratio =   4096 - 0) / (.25 - (-.25))
'            = 8.2 A-Dunits mv or 8192 A/Dunits/volt
'curratio= /(A/Dmax(=4096)-A/Dmin(=0))/(memcurmax(=12.5na)-memcurmin(=-12.5na))
CURRATIO# =  4096 - 0) / ((12.5 - (-12.5)) * .00000000014)
'            = 163.8 A/Dunits/na
'membranevolts=voltratio * membraneA/Dunits

'*****time calibration was measured from calibration routine below
sampint = .00301: ' there was .301 ms between samples
SMPDURTIM = .3: '   set sample interval to 300ms
npoints! = SMPDURTIM / sampint

WHEN$ = DATE$: REM Date$ string contains mm-dd-yyyy
MON$ = LEFT$(WHEN$, 2)
IF MON$ = "01" THEN MON$ = "JA"
IF MON$ = "02" THEN MON$ = "FE"
IF MON$ = "03" THEN MON$ = "MR"
IF MON$ = "04" THEN MON$ = "AP"
IF MON$ = "05" THEN MON$ = "MY"
IF MON$ = "06" THEN MON$ = "JN"
IF MON$ = "07" THEN MON$ = "JL"
IF MON$ = "08" THEN MON$ = "AU"
IF MON$ = "09" THEN MON$ = "SE"
IF MON$ = "10" THEN MON$ = "OC"
IF MON$ = "11" THEN MON$ = "NO"
IF MON$ = "12" THEN MON$ = "DE"
DAY$ = MID$(WHEN$, 4, 2)
YR$ = RIGHT$(WHEN$, 2)
WHEN$ = MON$ + DAY$ + YR$
CALL SOFTINIT
CALL INIT
```

```
' inch0=membrane voltage
' inch1=membrane current
' digin0=digital trigger to start sampling
slot4% = 4: slot6% = 6: chan0% = 0: chan1% = 1: chan2% = 2: chan3% = 3:
acc12% = 12: gain1% = 1: minus1% = -1
CALL IONMANA("inch0", slot6%, chan0%, acc12%, gain1%, minus1%)
CALL IONMANA("inch1", slot6%, chan1%, acc12%, gain1%, minus1%)
CALL IONMDIG("digin0", slot4%, chan0%, -1)

thl! = 2400: thh! = thl! + 10: 'threshold is in A/D units
euf% = -1: ' -1 means use A/D units
cy% = 0: ' 0 means don't cycle
sampint% = 10: 'samplint interval set fast as 570 system can sample

DIM volts%(npoints!), current%(npoints!), TICBUF%(5)

timcalflag = 0

20 CLS : SCREEN 2

PRINT " If want to calibrate time base, set timcalflag=1 in program."
 timcalflag = 0

80 PRINT
PRINT "1=depolarize to threshold"
PRINT "2=depolarize to two Xs threshold "
PRINT "3=hyperpolarize to 5mv"
PRINT "4=hyperpolarize to 15mv"
PRINT "5=hyperpolarize to 45mv"
INPUT "6=review data: ", n: PRINT

 IF n = 1 THEN descriptor$ = "1=depolarize to threshold": GOTO 90
 IF n = 2 THEN descriptor$ = "2=depolarize to two Xs threshold": GOTO 90
 IF n = 3 THEN descriptor$ = "3=hyperpolarize to 5mv": GOTO 90
 IF n = 4 THEN descriptor$ = "4=hyperpolarize to 15mv": GOTO 90
 IF n = 5 THEN descriptor$ = "5=hyperpolarize to 45mv": GOTO 90
 IF n = 6 THEN 2000
 GOTO 90

 90 CALL DIGINTEG("digin0", "on", -1, "st", "", cy%)
    CALL ANINQ("zvoltcurbuf%", npoints!, "inch0,inch1", sampint%, "wat":
    STIMTIME$ = TIME$

IF DEBUG = 1 THEN GOSUB 8300: 'printout of sampled values

150 ' draw box****************************************

n3! = 3: n1! = 1
wid1% = 1
minyval! = 1
maxyval! = 4095
point1! = 1
color1$ = "1"
widthlist1$ = "1"
color3$ = "3"
widthlist2$ = "2"

 SCREEN 1, 0: ' SCREEN 1 sets CGAcard to medium resolution mode(320x200)

MESSAGE$ = WHEN$ + " " + STIMTIME$
 ' LOCATE 24,1 selects the next to bottom line, 1st row, to write on
 LOCATE 24, 1: PRINT MESSAGE$ + ". " + descriptor$

 'plot data
 CALL GRAPH("zvoltcurbuf%",    widthlist1$    col ...
```

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```
reduce", -1, point1!, npoints!, euf%)
 CALL GRAPH("zvoltcurbuf%", widthlist2$, color3$, "pageo", minyval!, maxyval!,
reduce", -1, point1!, npoints!, euf%)

' plot tic marks
CALL ARMAKE("ZTICBUF%", n3!, wid1%, "")
TICBUF%(1) = 1: 'zero
TICBUF%(2) = 4096 / 2
TICBUF%(3) = 4096
 CALL ARPUTI("ZTICBUF%", n1', n3!, wid1%, "", TICBUF%())
 SCREEN 1
 CALL GRAPH("zticbuf%", widthlist1$, color1$, "PAGEO", minyval!, maxyval!, "norm
al", -1, n1!, n3!, euf%)
 CALL ARDEL("ZTICBUF%")

' LINE draws a box around area to graph
 LINE (7, 9)-(312, 168), 3, B

WHILE INKEY$ = ""
WEND

 CLS
 SCREEN 2
 INPUT "Replot? ", flag$: IF flag$ = "Y" OR flag$ = "y" THEN 150
 SCREEN 0

IF timcalflag = 1 THEN 8000: 'if calibrating time base, skip the rest
CALL arlastp("zvoltcurbuf%", lastp!): 'read in # points sampled
250 INPUT "Save data, (Y/N)?: ", Q$
 IF Q$ = "n" THEN 400
IF Q$ = "N" THEN 400
IF Q$ = "Y" THEN 260
IF Q$ = "y" THEN 260
GOTO 250
260 GOSUB 4000: 'define filename
GOSUB 4040: 'store data

400 CALL ARDEL("zvoltcurbuf%")
GOTO 20

2000 REM read data from disk*******************************
 PRINT "Read in data"
 GOSUB 4000: REM get filename
2010 INPUT " Enter: H to read response from hard disk, F from floppy disk.
, Q$
 IF Q$ = "H" OR Q$ = "h" THEN 2030
 IF Q$ = "F" OR Q$ = "f" THEN 2120
 GOTO 2020
2030 asciinf$ = "C:\OE40\DATA\" + SCIINF$
 ZZvoltCUR$ = "C:\OE40\DATA\" + ZvoltCUR$
 GOTO 2150

2120
 asciinf$ = "b:" + SCIINF$
 ZZvoltCUR$ = "B:" + ZCUR$

2160 OPEN asciinf$ FOR INPUT AS #1
 INPUT #1, STIMTIME$, ADDINFO$, descriptor$, SMPDURTIM, lastp!
 INPUT #1, voltratio, CURRATIO#, sampintX, adunitmin, adunitmax
 INPUT #1, advoltmin, advoltmax, npoints', stimdur
 INPUT #1, d1$, d2$, d3$, d4$, d5$, d6$, d7$, d8$, d9$, d10$, d11$
 INPUT #1, d12$, d13$, d14$
 INPUT #1, d1a$, d2a$, d3a$, d4a$, d5a$, d6a$, d7a$, d8a$, d9a$, d10a$, d11a$
 INPUT #1, d12a$, d13a$, d14a$
 INPUT #1, B1$, B2$, B3$, B4$, B5$, B6$, B7$, B8$, B9$, b10$, b11$
 INPUT #1, B1, B2, B3, B4, B5, B6, B7, B8, B9, b10, b11: CLOSE #1
```

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```
        CALL ARLOAD("zvoltcurbuf%", ZZvoltCUR$)
  PRINT d1$; d1a$
  PRINT d2$; d2a$
  PRINT d3$; d3a$
  PRINT d4$; d4a$
  PRINT d5$; d5a$
  PRINT d6$; d6a$
  PRINT d7$; d7a$
  PRINT d8$; d8a$
  PRINT d9$; d9a$
  PRINT d10$; d10a$
  PRINT d11$; d11a$
  PRINT d12$; d12a$
  PRINT d13$; d13a$
  PRINT d14$; d14a$
   INPUT n
  GOTO 150: 'graph data




4000 ' enter filename *****************************
 PRINT "Current date= "; WHEN$: INPUT "Want to change (Y/N)? ", flag$
 IF flag$ <> "Y" THEN 4015
4015 IF flag$ <> "y" THEN 4020
 PRINT "For date:JA=jan,FE=feb,MR=march,AP=april,MY=may,JN=june"
 PRINT "JL=july,AU=aug,SE=sept,OC=oct,NO=nov,DE=dec. For example:"
 PRINT "MR0586=March 5,86"
 INPUT "Enter new date: ", WHEN$
4020 INPUT "What's unit num (num 1=01, max=99)? ", unitnum$
 INPUT "What's run number (run 1=01, max=99)? ", RUNNUM$
 SCIINF$ = WHEN$ + unitnum$ + ".I" + RUNNUM$: REM ascii information
 ZvoltCUR$ = WHEN$ + unitnum$ + ".D" + RUNNUM$: REM zsoft voltage & current data
RETURN



4040 'store data*****************
INPUT "Additional description NO COMMAS (255 CH MAX): ", ADDINFO$
 V = LEN(ADDINFO$)
 IF V > 255 THEN PRINT "Message "; V - 255; " ch. too long.": GOTO 4040
asciinf$ = "C:\qb40\DATA\" + SCIINF$
ZZvoltCUR$ = "C:\qb40\DATA\" + ZvoltCUR$

4060 OPEN asciinf$ FOR OUTPUT AS #1
 WRITE #1, STIMTIMES$, ADDINFO$, descriptors$, SMPDURTIM, lastp!
 WRITE #1, voltratio, CURRATIO#, sampint%, adunitmin, adunitmax
 WRITE #1, advoltmin, advoltmax, npoints!, stimdur
 WRITE #1, d1$, d2$, d3$, d4$, d5$, d6$, d7$, d8$, d9$, d10$, d11$
 WRITE #1, d12$, d13$, d14$
 WRITE #1, d1a$, d2a$, d3a$, d4a$, d5a$, d6a$, d7a$, d8a$, d9a$, d10a$, d11a$
 WRITE #1, d12a$, d13a$, d14a$
 WRITE #1, B1$, B2$, B3$, B4$, B5$, B6$, B7$, B8$, B9$, b10$, b11$
 WRITE #1, B1, B2, B3, B4, B5, B6, B7, B8, B9, b10, b11: CLOSE #1

CALL ARLABEL("zvoltcurbuf%", descriptor$)
CALL ARSAVE("zvoltcurbuf%", ZZvoltCUR$)

 INPUT "Make a backup on floppy disk? ", Q$:
 IF Q$ = "N" OR Q$ = "n" THEN 4090
  INPUT "Insert floppy disk in drive A  and hit ENTER", Q$
  asciinf$ = "A:" + SCIINF$
  ZZvoltCUR$ = "A:" + ZvoltCUR$
  GOTO 4060
4090 RETURN

8000 '***************TIME CALIBRATION ROUTINES
```

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```
      CALL ARLOAD("zvoltcurbuf%", ZZvoltCUR$)
    PRINT d1$; d1a$
    PRINT d2$; d2a$
    PRINT d3$; d3a$
    PRINT d4$; d4a$
    PRINT d5$; d5a$
    PRINT d6$; d6a$
    PRINT d7$; d7a$
    PRINT d8$; d8a$
    PRINT d9$; d9a$
    PRINT d10$; d10a$
    PRINT d11$; d11a$
    PRINT d12$; d12a$
    PRINT d13$; d13a$
    PRINT d14$; d14a$
     INPUT n
     GOTO 150: 'graph data




    4000 ' enter filename *****************************
     PRINT "Current date= "; WHEN$: INPUT "Want to change (Y/N)? ", flag$
     IF flag$ <> "Y" THEN 4015
    4015 IF flag$ <> "y" THEN 4020
     PRINT "For date:JA=jan,FE=feb,MR=march,AP=april,MY=may,JN=june"
     PRINT "JL=july,AU=aug,SE=sept,OC=oct,NO=nov,DE=dec. For example:"
     PRINT "MR0586=March 5,86"
     INPUT "Enter new date: ", WHEN$
    4020 INPUT "What's unit num (num 1=01, max=99)? ", unitnum$
     INPUT "What's run number (run 1=01, max=99)? ", RUNNUM$
     SCIINF$ = WHEN$ + unitnum$ + ".I" + RUNNUM$: REM ascii information
     ZvoltCUR$ = WHEN$ + unitnum$ + ".D" + RUNNUM$: REM zsoft voltage & current data
    RETURN



    4040 'store data******************
    INPUT "Additional description NO COMMAS (255 CH MAX): ", ADDINFO$
     V = LEN(ADDINFO$)
     IF V > 255 THEN PRINT "Message "; V - 255; " ch. too long.": GOTO 4040
    asciiinf$ = "C:\qb40\DATA\" + SCIINF$
    ZZvoltCUR$ = "C:\qb40\DATA\" + ZvoltCUR$

    4060 OPEN asciiinf$ FOR OUTPUT AS #1
     WRITE #1, STIMTIME$, ADDINFO$, descriptor$, SMPDURTIM, lastp!
     WRITE #1, voltratio, CURRATIO#, sampint%, adunitmin, adunitmax
     WRITE #1, advoltmin, advoltmax, npoints!, stimdur
     WRITE #1, d1$, d2$, d3$, d4$, d5$, d6$, d7$, d8$, d9$, d10$, d11$
     WRITE #1, d12$, d13$, d14$
     WRITE #1, d1a$, d2a$, d3a$, d4a$, d5a$, d6a$, d7a$, d8a$, d9a$, d10a$, d11a$
     WRITE #1, d12a$, d13a$, d14a$
     WRITE #1, B1$, B2$, B3$, B4$, B5$, B6$, B7$, B8$, B9$, b10$, b11$
     WRITE #1, B1, B2, B3, B4, B5, B6, B7, B8, B9, b10, b11: CLOSE #1

    CALL ARLABEL("zvoltcurbuf%", descriptor$)
    CALL ARSAVE("zvoltcurbuf%", ZZvoltCUR$)

    INPUT "Make a backup on floppy disk? ", Q$:
    IF Q$ = "N" OR Q$ = "n" THEN 4090
     INPUT "Insert floppy disk in drive A  and hit ENTER", Q$
     asciiinf$ = "A:" + SCIINF$
     ZZvoltCUR$ = "A:" + ZvoltCUR$
     GOTO 4060
    4090 RETURN

    8000 '***************TIME CALIBRATION ROUTINES
```

```basic
CALL argeti("zvoltcurbuf%", 1!, npoints!, widthlist1$, "", volts%())
CALL argeti("zvoltcurbuf%", 1!, npoints!, widthlist2$, "", current%())
CALL arlastp("zvoltcurbuf%", lastp!)


PRINT "keithley array sample size: "; lastp!
PRINT "Specified sample size: "; npoints!
INPUT n

IF DEBUG = 0 THEN 8100

FOR i = 1 TO 50
PRINT i, volts%(i), current%(i)
NEXT i

threshold = 2500
curcount = 0
voltcount = 0
flag = 0

8100 FOR i = 1 TO lastp!
' PRINT i, volts%(i); "   "; threshold: INPUT n

IF volts%(i) < threshold THEN 8150
IF flag = 1 THEN 8160
voltcount = voltcount + 1
' PRINT "voltcount: ", voltcount: INPUT n
flag = 1
GOTO 8160
8150 flag = 0
8160 NEXT i

flag = 0
8200 FOR i = 1 TO lastp!
IF current%(i) < threshold THEN 8250
IF flag = 1 THEN 8260
curcount = curcount + 1
flag = 1
GOTO 8260
8250 flag = 0
8260 NEXT i
n = npoints!
PRINT "curcount: "; curcount
PRINT "voltcount: "; voltcount
INPUT "Enter time between impulses (ms): ", interval
PRINT "current trace: number of samples between pulses: "; n / curcount
PRINT "voltage trace: number of samples between pulses: "; n / voltcount
PRINT "from current trace: time between samples: "; interval / (n / curcount)
PRINT "from voltage trace: time between samples: "; interval / (n / voltcount)
INPUT n
RETURN


8300 : 'debug printout of sampled values********************
max! = 30
PRINT "will display "; max!; " points"
value0! = 0
value1! = 0
FOR dep! = 1 TO max!
DEP1! = dep! + npoints!
CALL argetvalf("zvoltcurbuf%", dep!, -1, "inch0,inch1", value0!, euf%)
CALL argetvalf("zvoltcurbuf%", DEP1!, -1, "inch0,inch1", value1!, euf%)
PRINT dep!, value0!, DEP1!, value1!
NEXT dep!
INPUT n
RETURN                                    45
```